Form PTO-1390 (REV 10-95) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		702-010411
		U.S. APPLICATION NO (If known, see 37 CFR 1.5) 09/806178
INTERNATIONAL APPLICATION NO PCT/EP99/07405	INTERNATIONAL FILING DATE 24.09.99 (24 September 1999)	PRIORITY DATES CLAIMED 29.09.98 (29 September 1998) 22.06.99 (22 June 1999)

TITLE OF INVENTION **USE OF COMPOUNDS THAT REDUCE ALPHA2-ANTIPLASMIN IN VIVO FOR THE PREPARATION OF A COMPOSITION FOR THE TREATMENT OF ISCHEMIC STROKE**

APPLICANT(S) FOR DO/EO/US

Nagia NOBUO and Désiré José COLLEN

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U S.C. 371

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U S C 371.

3. ☒ This express request to begin national examination procedures (35 U S C 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U S C 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date

5. ☒ A copy of the International Application as filed (35 U S C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau)
   b. ☒ has been transmitted by the International Bureau
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☐ A translation of the International Application into English (35 U S C 371(c)(2))

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U S C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau)
   b. ☐ have been transmitted by the International Bureau
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S C 371(c)(3))

9. ☐ An oath or declaration of the inventor(s) (35 U S C 371(c)(4))

10 ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11 ☐ An Information Disclosure Statement under 37 CFR 1 97 and 1 98

12 ☐ An assignment document for recording A separate cover sheet in compliance with 37 CFR 3 28 and 3 31 is included.

13 ☒ A FIRST preliminary amendment

☐ A SECOND or SUBSEQUENT preliminary amendment

14 ☐ A substitute specification

15 ☐ A change of power of attorney and/or address letter.

16 ☒ Other items or information
   a. WO 00/18436-Front Page with Abstract, Specification, Claims, Drawings and Search Report (25 pp.)
   b. International Preliminary Examination Report and Annex (8 pp.)

U.S APPLICATION NO (If known, see 37 CFR 1.5) 09/806178	INTERNATIONAL APPLICATION NO PCT/EP99/07405	ATTORNEY'S DOCKET NUMBER 702-010411

17. ☒ The following fees are submitted:

		CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO	$860.00	
International preliminary examination fee paid to USPTO (37 CFR 1 482)	$690.00	
No international preliminary examination fee paid to USPTO (37 CFR 1 482) but international search fee paid to USPTO (37 CFR 1 445(a)(2)) . .	$710.00	
Neither international preliminary examination fee (37 CFR 1 482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO	$1,000.00	
International preliminary examination fee paid to USPTO (37 CFR 1 482) and all claims satisfied provisions of PCT Article 33(2)-(4). . . .	$100.00	

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1 492(e))	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	6 - 20	0	X **$18.00**	$ 0.00	
Independent claims	1 - 3 =	0	X **$80.00**	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$ 990.00	
Reduction of 1/2 for filing by small entity, if applicable Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1 27, 1 28).				$ 0.00	
SUBTOTAL =				$ 990.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				$ 0.00	
TOTAL NATIONAL FEE =				$ 990.00	
Fee for recording the enclosed assignment (37 CFR 1 21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3 28, 3 31) **$40.00** per property +				$ 0.00	
TOTAL FEES ENCLOSED =				$ 990.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ **990.00** to cover the above fees is enclosed

b. ☐ Please charge my Deposit Account No _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Assistant Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No 23-0650. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

PATENT APPLICATION/PCT
Atty. Docket No. 702-010411

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	
Nagia NOBUO Désiré José COLLEN	: :	USE OF COMPOUNDS THAT REDUCE ALPHA2-ANTIPLASMIN IN VIVO FOR THE PREPARATION
International Application No. PCT/EP99/07405	: :	OF A COMPOSITION FOR THE TREATMENT OF ISCHEMIC STROKE
International Filing Date 24 September 1999	: :	
Priority Dates Claimed 29 September 1998 22 June 1999	: : :	
Serial No. Not Yet Assigned	:	
Filed Concurrently Herewith	:	

Pittsburgh Pennsylvania
March 28, 2001

PRELIMINARY MENDMENT

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-identified patent application as follows:

IN THE SPECIFICATION:

Please insert section headings specification paragraphs as follows.

On page 1, after the title, please insert the following section headings:

BACKGROUND OF THE INVENTION

1. Field of the Invention

On page 1, after the first complete paragraph, please insert the following section heading:

2. Description of the Related Art

After the paragraph bridging pages 1 and 2, please insert the following section headings and paragraphs:

SUMMARY OF THE INVENTION

The invention relates to a new means for treating ischemic stroke. It is an object of this invention to treat focal cerebral ischemic infarction by administering at least one compound that reduces α_2-antiplasmin *in vivo* in the form of a therapeutical composition, so that the size of the focal cerebral ischemic infarct is reduced. Compounds of the invention reducing α_2-antiplasmin concentration or activity are, for example, plasmin, mini-plasmin (lacking the first four kringles) and micro-plasmin (lacking all five kringles). Also suitable for reducing α_2-antiplasmin concentration or activity are neutralizing antibodies, such as monoclonal antibodies, and derivatives of neutralizing antibodies, such as Fab fragments and scFv fragments.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph of infarct volume plotted relative to amounts of plasminogen system components;

Fig. 2 is a graph of infarct volume plotted relative to adenoviral transfer of t-PA or PAI-1 genes; and

Fig. 3 is a graph of infarct volume plotted relative to (A) α_2-AP genotype and (B) injection of hα_2-AP or of h$\alpha\alpha_2$-AP followed by anti-hα_2-AP fragments.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

IN THE CLAIMS:

Original claim 1 was amended during Chapter II proceedings by substituting new claim 1 in a letter dated November 24, 2000. Please cancel original claims 1-6 and cancel amended claim 1 and rewrite them as new claims 7-12 as follows:

7. A method for the treatment of focal cerebral ischemic infarction by administering at least one compound that reduces α_2-antiplasmin *in vivo* in the form of a therapeutical composition, whereby the size of the focal cerebral ischemic infarct is reduced.

8. The method according to claim 7, wherein the at least one compound reduces the circulating α_2-antiplasmin concentration.

9. The method according to claim 7, wherein the at least one compound reduces the circulating α_2-antiplasmin activity.

10. The method according to claim 7, wherein the at least one compound is selected from the group consisting of α_2-antiplasmin neutralizing antibodies and derivatives thereof.

11. The method according to claim 10, wherein the derivatives are selected from the group consisting of Fab fragments and ScFv fragments.

12. The method according to claim 7, wherein the at least one compound is an α_2-antiplasmin neutralizing compound selected from the group consisting of plasmin, mini-plasmin (lacking the first four kringles) or micro-plasmin (lacking all five kringles).

IN THE ABSTRACT:

After the claims, please insert a page containing the Abstract Of The Disclosure, which is attached hereto as a separately typed page.

REMARKS

The specification has been amended to place the application in conformance with standard United States patent practice.

Examination and allowance of pending claims 7-12 are respectfully requested.

Respectfully submitted,

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USE OF COMPOUNDS THAT REDUCE ALPHA2-ANTIPLASMIN IN VIVO FOR THE PREPARATION OF A COMPOSITION FOR THE TREATMENT OF ISCHEMIC STROKE

ABSTRACT OF THE DISCLOSURE

The present invention relates to a new means for the treatment of focal ischemic cerebral infarction (ischemic stroke). It has been found that reduction of α_2-antiplasmin leads to a significantly smaller focal cerebral infarct size. The invention therefore provides the use of compounds that reduce α_2-antiplasmin concentration or activity *in vivo*, for the preparation of a therapeutical composition for the treatment of focal cerebral ischemic infarction (ischemic stroke).

USE OF COMPOUNDS THAT REDUCE ALPHA2-ANTIPLASMIN IN VIVO FOR THE PREPARATION OF A COMPOSITION FOR THE TREATMENT OF ISCHEMIC STROKE

The present invention relates to a new means for the treatment of focal ischemic cerebral infarction (ischemic stroke).

Focal ischemic cerebral infarction occurs when the arterial blood flow to a specific region of the brain is reduced below a critical level resulting in neuronal cell death. It is thought that neuronal degeneration in central nervous system (CNS) diseases such as stroke, epilepsy and Alzheimer's disease is stimulated by an excess of the excitatory amino acid glutamate (2). Injection of glutamate agonists in the CNS indeed induces hippocampal neuronal cell death similar to that observed in neurodegenerative diseases (3).

Excitotoxin-induced neuronal degeneration is mediated by tissue-type plasminogen activator (t-PA) (4). Consistent with this observation, mice deficient in t-PA are resistant to, and infusion of plasminogen activator inhibitor-1 (PAI-1) protects against excitotoxin-mediated hippocampal neuronal degeneration (4-6).

Furthermore, deficiency of plasminogen (Plg), the zymogen substrate of t-PA, and infusion of α_2-antiplasmin (α_2-AP), protect mice against excitotoxin-induced hippocampal neuronal death (5). It has been proposed that plasmin-mediated degradation of laminin sensitizes hippocampal neurons to cell death by disrupting neuron-extracellular matrix interaction (7).

Wang et al. (8) recently demonstrated that neuronal damage after focal cerebral ischemia induced by transient occlusion of the middle cerebral artery was also reduced in mice with t-PA deficiency and exacerbated by t-PA infusion. This suggests that the plasminogen system may be involved both in establishing a cerebral ischemic infarct and in its extension during thrombolytic therapy. It was recently demonstrated that the neurotoxic effect of t-PA on persistent focal cerebral ischemia also occurred with other thrombolytic agents, including

streptokinase and staphylokinase (9). Thus, in those patients with persistent cerebral arterial occlusion, thrombolytic therapy for ischemic stroke may cause infarct extension, which would not only partially offset the established overall beneficial effect of arterial recanalization (10, 11), but indeed be harmful to a subgroup of patients. Because it is not possible to distinguish between patients who will and those who will not achieve cerebral arterial recanalization with thrombolytic therapy, the development of specific conjunctive strategies to counteract the neurotoxic effects of thrombolytic agents on persisting focal cerebral ischemia appear to be warranted.

It is therefore the object of the present invention to provide a new means for treating ischemic stroke.

In the research that led to the present invention the following was contemplated. Although it is assumed that neuronal injury during focal ischemia in the brain occurs primarily as a result of accumulation of excitotoxins such as glutamates, the role of plasmin-mediated laminin degradation or alternative mechanisms in the pathogenesis of cortical neuronal cell death has not been demonstrated. In order to delineate the contribution of individual components of the plasminogen (fibrinolytic) system on focal cerebral ischemic infarction, the present inventors then quantitated infarct size produced by ligation of the left middle cerebral artery (MCA) in mice with targeted inactivation of the genes encoding Plg, its activators tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA), or the fibrinolytic inhibitors PAI-1 or α_2-AP. In addition, the effects of adenoviral transfer of the t-PA and PAI-1 genes and of infusion of human α_2-AP on cerebral infarction were studied.

Whereas the findings of Strickland et al., that t-PA deficiency protects against focal cerebral ischemic

infarction were fully confirmed, and extended by the observation that PAI-1 deficiency resulted in significantly larger infarct sizes, the observation that Plg deficiency protects against excitotoxin induced neuronal cell death could not be confirmed. Instead it was found that focal cerebral infarct size was significantly larger in mice with Plg deficiency and conversely, significantly smaller in mice with α_2-AP deficiency.

In aggregate, these findings indicate that plasminogen system components affect focal cerebral ischemic infarct size at two different levels: 1) reduction of t-PA activity (t-PA gene inactivation or PAI-1 gene transfer) reduces, while its augmentation (t-PA gene transfer or PAI-1 gene inactivation) increases infarct size, and 2) reduction of Plg activity (Plg gene inactivation or α_2-AP injection) increases, while its augmentation (α_2-AP gene inactivation or α_2-AP neutralization) reduces infarct size. The findings are incompatible with a unique linked pathway in which t-PA-mediated plasmin generation would lead to neuronal cell death, but suggests two independent (t-PA mediated and Plg-mediated, respectively) mechanisms operating in opposite direction.

The internally consistent observations with α_2-AP were unexpected but are most relevant for the treatment of ischemic stroke. Firstly a correlation was found between infarct size and genotype with heterozygotes displaying infarct sizes between those of the wild type and homozygous phenotypes. Secondly, bolus injection of human α_2-AP (hα_2-AP) in α_2-AP$^{-/-}$ mice caused a dose-related infarct expansion. Importantly, Fab fragments from affino-specific polyclonal rabbit anti-hα_2-AP antibodies, given intravenously 40 min after occlusion of the MCA, significantly reduced the cerebral ischemic infarct size. This observation suggests a potential avenue to counteract focal ischemic infarction with the use of α_2-AP inhibitors (e.g. neutralizing monoclonal antibodies or compounds neutralizing α_2-AP

activity). This approach was confirmed by infusion of plasmin in mice with MCA occlusion which, by neutralizing α_2-AP, significantly reduced infarct size. The concentration of α_2-AP in human plasma is 1 mM (12), corresponding to a total body pool of approximately 500 mg. An equivalent dose of a monoclonal Fab fragment would be approximately 400 mg, and that of plasmin approximately 500 mg, which is high but not excessive for single therapeutic administration. Furthermore, the observation that infarct size is proportional to the α_2-AP level (derived from the gene dose effect and the dose-response) suggests that a partial reduction of the plasma level might have a significant beneficial effect.

In view of the above the invention thus relates to the use of compounds that reduce α_2-AP activity in vivo for the treatment of focal cerebral ischemic infarction (ischemic stroke).

In a specific embodiment of the invention use is made of compounds that reduce the circulating α_2-AP concentration. A lower concentration of α_2-AP will lead to a lower activity. In an alternative embodiment, the activity of circulating α_2-AP is reduced directly.

Compounds that are suitable for the reduction of α_2-AP concentration and activity are for example α_2-AP neutralizing antibodies or derivatives thereof. Preferred antibodies are monoclonal antibodies. Derivatives are preferably Fab fragments, scFv fragments.

Compounds neutralizing α_2-AP are for example plasmin, mini-plasmin (lacking the first 4 kringles) or micro-plasmin (lacking all five kringles).

The present invention will be demonstrated in more detail in the following examples, which are however not intended to be limiting to the scope of the invention. In the examples reference is made to the following drawings:

Figures 1 to 3 are histograms comparing the volume (in mm^3) of focal cerebral ischemic infarcts after ligation of the middle cerebral artery (MCA) in mice. The

data represent mean values and the vertical bars SEM, with the number of experiments given in the columns.

Figure 1 shows the effect of deficiency of plasminogen system components (genotype in abscissa) on focal ischemic cerebral infarct size (in mm^3). WT: wild type (pooled values of 50% C57BL6/50% S129, 100% C57BL6 and 100% S129 genetic background).

Figure 2 shows the effect of adenoviral transfer of the t-PA or PAI-1 genes on focal ischemic cerebral infarct size in t-PA or PAI-1 deficient mice, respectively.

Figure 3 shows the effect of α_2-AP on focal ischemic cerebral infarct size.

A. Effect of α_2-AP genotype on cerebral infarct size.

B. Effect of injection of hα_2-AP or of h$\alpha\alpha_2$-AP followed by anti-hα_2-AP Fab fragments on cerebral infarct size.

EXAMPLES

EXAMPLE 1

Murine cerebral ischemic infarction model

1. Introduction

All mice included in the present study were generated and bred at the Specific Pathogen Free Facility of the Center for Transgene Technology and Gene Therapy, Campus Gasthuisberg, K.U. Leuven. Gene inactivation was obtained by homologous recombination in embryonic stem cells targeting the genes encoding tissue-type plasminogen activator (t-PA) (13), urokinase-type plasminogen activator (u-PA) (13), plasminogen activator inhibitor-1 (PAI-1) (14, 15), plasminogen (Plg) (16), or α_2-antiplasmin (α_2-AP) (17), as previously described. Mice with inactivated genes encoding u-PA receptor (u-PAR) (18) were not included because of the normal results obtained with u-PA deficient mice.

2. Materials and methods

2.1 Materials

Human α_2-AP was prepared from fresh frozen plasma as previously described (19).

Polyclonal antisera were raised in rabbits by subcutaneous injection of 200 mg purified human α_2-AP suspended in complete Freund's adjuvant, followed at two biweekly intervals by injection of the antigen suspended in incomplete Freund's adjuvant. Serum was obtained by repeated ear vein puncture. Pooled sera were chromatographed on Protein-A Sepharose (0.5 ml serum per ml wet gel), equilibrated with 0.1 M Tris.HCl, pH 8.1 and IgG eluted with 0.1 M glycine.HCl, pH 2.8, yielding approximately 10 mg protein per ml serum. Affino-specific antibodies were obtained from the dialyzed IgG pool by chromatography on a CNBr-activated Sepharose column substituted with human α_2-AP (2.5 mg/ml wet gel) and eluted with 0.1 M glycine.HCl, pH 2.8, yielding approximately 0.1 mg specific IgG per mg applied.

Fab fragments were obtained from the affino-specific IgG by digestion with papain. Therefore IgG was dissolved to a concentration of 5 mg/ml and digested with 1 percent (w/w) papain in the presence of 50 mM cysteine, 1 mM EDTA, 0.1 M phosphate buffer, pH 7.0 for 5 hours. The reaction was arrested by addition of iodoacetamide to a final concentration of 75 mM. After dialysis the mixture was purified on a protein A Sepharose column equilibrated with PBS. Fab concentration was determined by ELISA calibrated against an IgG standard. SDS gel electrophoresis essentially revealed homogeneous Fab fragments (not shown).

2.2 Production of adenoviral vectors

The recombinant adenoviruses AdCMVt-PA and AdCMVPAI-1 were generated by homologous recombination in 293 cells essentially as previously described (20). For AdCMVt-PA, an XbaI-fragment of the plasmid pSTEt-PA encoding wild type human t-PA was ligated into

XbaI-digested pACCMVpLpA (21) to produce pACCMVt-PA. The adenovirus precursor pACCMVPAI-1 was generated by ligating the 1.4-kb EcoRI/BglII fragment of pPAI-1RBR containing the entire coding sequence of human PAI-1 into EcoRI/BamHI-digested pACCMVpLpA. In these plasmids, the t-PA and PAI-1 cDNA are positioned between the human cytomegalovirus immediate-early enhancer/promoter and the SV40 t-antigen intron/polyadenylation signal to form a complete transcriptional unit.

Monolayer cultures of 293 cells (22) were cotransfected with 10 mg of pACCMVt-PA or pACCMVPAI-1 and 5 mg of pJM17 (20), a plasmid containing a full-length adenovirus 5 dl309 genome. Homologous recombination between these plasmids results in the formation of recombinant viral genomes in which the adenovirus E1 region is replaced by the respective t-PA or PAI-1 transgenes. Replication of the recombinant viruses in cultured 293 cells is supported by E1A gene products supplied in trans from a copy of E1 integrated into the 293 cell genome.

After transfection, recombinant viral plaques were harvested and amplified as described (23). The identity of recombinant viruses was determined by restriction analysis and Southern blotting of viral DNA prepared from productively infected 293 cells. The recombinant adenovirus AdRR5, which lacks an inserted gene in the E1 position, was generated from pACRR5 and pJM17 in the same manner and was used as a control adenovirus (24, 25). Recombinant viruses were replaqued to ensure clonal identity before further use. Large scale production of recombinant adenovirus was performed as described (23). Purified virus was supplemented with 0.1 mg/ml sterile bovine serum albumin (BSA), snap frozen in liquid nitrogen and stored at -80°C until use. The titer of infectious viral particles in purified stocks was determined by plaque assay on monolayers of 293 cells with 1 hour of adsorption at 37°C. Purified viral stocks of $>10^{10}$ plaque forming units (pfu) per ml were routinely

obtained. The kinetics and organ distribution of t-PA and PAI-1 expression following adenoviral transfer by intravenous bolus injection have been described elsewhere (26, 27).

2.3 Preparation of human plasmin

Human plasminogen was prepared from fresh frozen human blood bank plasma, essentially as described previously (28). Human plasma (6 liter), to which 20 units aprotinin (Trasylol, Bayer, Germany) was added per ml, was cleared by centrifugation at 4,000 rpm for 15 min at 4°C. Lysine-Sepharose (200g wet weight, substitution level approximately 1 mg lysine per g wet Sepharose gel) was added to the supernatant, the mixture stirred for 1 hour at 4°C and the gel recovered on a Buchner funnel. Then 120 g Lysine-Sepharose was added to the filtrate, the mixture stirred and the gel recovered as above. The combined gel fractions were washed with 18 liter 0.2 M K_2HPO_4/KH_2PO_4 buffer, pH 7.5, containing 10 units aprotinin per ml, then poured into a 5 x 60 cm column and washed with 0.02 M NaH_2PO_4, 0.1 M NaCl buffer, pH 7.5, containing 10 units/ml aprotinin at 4°C until the absorbance of the wash fluid at 280 nm was less than 0.05. The column was then eluted with wash buffer containing 0.05 M 6-aminohexanoic acid and protein containing fractions pooled. From 6 liter plasma approximately 145 ml fluid containing 650 mg protein was obtained. The pool was concentrated 2.5-fold on an Amicon PM10 filter and gel filtered on a 5 x 90 cm column of ultragel AcA44 equilibrated with 0.02 M NaH_2PO_4, 0.1 M NaCl buffer, pH 7.5, at a rate of 60 ml per hour. The main peak, containing approximately 590 mg protein was concentrated on an Amicon PM10 filter to a concentration of 10 mg/ml and frozen until use.

Human plasmin was prepared from plasminogen as follows. Lysine-Sepharose (20 g wet gel) was added to human plasminogen (200 mg) solution, the mixture stirred for 3 hours at 4°C, the gel washed on a Buchner funnel

and resuspended in 30 ml 0.1 M NaH_2PO_4 buffer, pH 7.4. Urokinase (500 µl of a 50 µM solution, prepared by activation of Saruplase (Grünenthal, Aachen, Germany) with Plasmin.Sepharose was added and the mixture stirred for 15 hours at 4°C. The gel was then washed on a Buchner funnel with 0.1 M NaH_2PO_4 buffer, pH 7.4, poured into a 1.5 x 16 cm column, washed with 0.1 M NaH_2PO_4 buffer, pH 7.4 until the absorbance at 280 nm of the wash fluid was less than 0.05, and eluted with 0.1 M NaH_2PO_4 buffer containing 0.05 M 6-aminohexanoic acid. The protein containing fractions were pooled, glycerol was added to a final concentration of 10 percent and the pool was dialyzed at 4°C against 0.1 M NaH_2PO_4 buffer containing 10 percent glycerol. The final recovery was 25 ml solution with a protein concentration of 4.0 mg/ml and an active plasmin concentration of 25 µM.

2.4 Measurement of α_2-antiplasmin in plasma

α_2-Antiplasmin levels in murine plasma were measured by a chromogenic substrate assay, based on its rapid inhibition of plasmin (29). Briefly 10 µl mouse plasma (diluted 1/10 in 0.05 M NaH_2PO_4 buffer, pH 7.4, containing 0.01% Tween 20) is mixed at 37°C with 420 µl 0.05 Tris HCl, 0.1 M NaCl buffer, pH 7.4, containing 0.01% Tween 20, and with 20 µl of 0.125 µM human plasmin (final concentration 5 nM). After 10s incubation, 50 µl of 3 mM S2403 (Chromogenics, Antwerp, Belgium) is added and the change in absorbance measured at 405 nm. The change in absorbance is approximately 0.18 min^{-1} with buffer and 0.09 min^{-1} with pooled murine plasma.

2.5 Animal experiments

Animal experiments were conducted according to the guiding principles of the American Physiological Society and the International Committee on Thrombosis and Haemostasis (30).

Focal cerebral ischemia was produced by persistent occlusion of the MCA according to Welsh et al. (31). Briefly, mice of either sex, weighing 20 to 30 g, were anesthetized by intraperitoneal injection of ketamine (75 mg/ml, Apharmo, Arnhem, The Netherlands) and xylazine (5 mg/ml, Bayer, Leverkusen, Germany). Atropine (1 mg/kg; Federa, Brussels, Belgium) was administered intramuscularly, and body temperature was maintained by keeping the animals on a heating pad. A "U" shape incision was made between the left ear and left eye. The top and backside segments of the temporal muscle were transsected and the skull was exposed by retraction of the temporal muscle. A small opening (1 to 2 mm diameter) was made in the region over the MCA with a hand-held drill, with saline superfusion to prevent heat injury. The meningae were removed with a forceps and the MCA was occluded by ligation with 10-0 nylon thread (Ethylon, Neuilly, France) and transsected distally to the ligation point. Finally, the temporal muscle and skin were sutured back in place.

AdCMVt-PA, AdCMVPAI-1 or AdRR5 were given as an intravenous bolus of 1.3×10^9 plaque forming units (p.f.u.) 4 days before ligation of the MCA. Human α_2-AP ($h\alpha_2$-AP) was given intravenously divided in 2 injections, given 1 min before and 30 min after ligation of the MCA, respectively. Fab fragments were injected intravenously as a bolus, 10 min after the second $h\alpha_2$-AP injection. Human plasmin was given intravenously as a bolus, either 15 min before or 15 min after ligation of the MCA.

The animals were allowed to recover and were then returned to their cages. After 24 hours, the animals were sacrificed with an overdose of Nembutal (500 mg/kg, Abbott Laboratories, North Chicago, IL) and decapitated. The brain was removed and placed in a matrix for sectioning in 1 mm segments. The sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline (32), incubated for 30 min at 37°C, and placed in 4 % formalin in phosphate buffered saline. With this

procedure, the necrotic infarct area remains unstained (white) and is clearly distinguishable from stained (brick red) viable tissue. The sections were photographed and subjected to planimetry. The infarct volume was defined as the sum of the unstained areas of the sections multiplied with their thickness.

The data are represented as mean ± SEM of n determinations. The significance of differences was determined using analysis of variance followed by Fisher's PLSD test, using the Statview software package or by Student's t-test.

EXAMPLE 2

Cerebral ischemic infarct size in mice with targeted inactivation of genes encoding plasminogen system components

Ligation of the left MCA induced a cerebral infarct with a volume of 7.6 ± 1.1 mm^3 (n= 11) in wild type mice with a mixed (50%) S129 and (50%) C57BL/6 genetic background, of 9.3 ± 2.7 mm^3 (n= 6) in inbred C57BL/6 mice and of 6.4 ± 1.3 mm^3 (n= 6) in inbred S129 mice (p= NS versus mixed background, results not shown).

Inactivation of the t-PA gene was associated with a significant reduction of infarct size to 2.6 ± 0.80 mm^3 (n= 11), (p< 0.0001 vs wild type mice), whereas inactivation of the u-PA gene had no effect on infarct size (7.8 ± 1.0 mm^3, n= 8, p= NS vs wild type).

Inactivation of the PAI-1 gene was associated with a significant increase in infarct size (16 ± 0.52 mm^3, n= 6, p< 0.0001 vs wild type) (Figure 1). In mice with inactivated Plg genes, cerebral infarct size was significantly larger than in wild type mice (12 ± 1.2 mm^3, n=9, p=0.037 vs wild type), whereas, conversely, in α_2-AP gene deficient mice, infarct size was markedly reduced (2.2 ± 1.1 mm^3, n= 7, p= 0.0001 vs wild type) (Figure 1).

EXAMPLE 3

Effect of t-PA and PAI-1 gene transfer on cerebral infarct size

Injection of 1.3×10^9 p.f.u. of AdCMVt-PA in 6 t-PA$^{-/-}$ mice 4 days before MCA ligation was associated with a cerebral infarct size of 6.0 ± 1.3 mm^3, significantly larger than the infarcts in 5 t-PA$^{-/-}$ mice injected with the control virus AdRR5 (1.8 ± 0.63, $p= 0.028$) (Figure 2A). Conversely, injection of 1.3×10^9 p.f.u. of AdCMVP-AI-1 in 5 PAI-1$^{-/-}$ mice was associated with a cerebral infarct size of 10 ± 1.4 mm^3, significantly smaller than the infarcts in 5 PAI-1$^{-/-}$ mice injected with the control virus AdRR5 (13 ± 1.0 mm^3, $p= 0.019$) (Figure 2B).

EXAMPLE 4

Effect of α_2-antiplasmin on cerebral infarct size

Cerebral infarct size correlated with α_2-AP gene dose, corresponding to 11 ± 2.0, 4.9 ± 2.0 and 2.2 ± 1.1 mm^3 in wild type, heterozygous and homozygous deficient mice, respectively (Figure 3A). Injection of human α_2-AP in groups of 4 α_2-AP$^{-/-}$ mice increased the infarct size to 13 ± 2.5 mm^3 ($n= 4$) with a 1 mg total dose and to 11 ± 1.5 mm^3 ($n= 6$) with a 0.2 mg total dose. Injection of 1.7 mg affino-specific Fab against human α_2-AP in mice given 0.2 mg human α_2-AP reduced the cerebral infarct size to 5.1 ± 1.1 mm^3 ($n= 7$, $p= 0.0040$ vs 0.2 mg human α_2-AP) (Figure 3B).

The above examples show that reduction of α_2-AP activity (reduced α_2-AP gene expression or reduction of circulating α_2-AP with inhibitors) reduces focal cerebral ischemic infarct size, such as encountered during ischemic stroke.

EXAMPLE 5

Effect of plasmin on cerebral infarct size

Injection of 50, 100 or 150 μg human plasmin (Pli) in mice weighing approximately 30 g decreased the α_2-AP levels in blood samples taken after 30 s to 67, 40 and 31 percent of baseline, respectively (mean of 2 mice, with less than 15 percent variability). Injection of 200 μg Pli in 3 mice reduced the plasma α_2-AP levels to 59 ± 4.8, 67 ± 4.4 and 70 ± 2.5 percent after 2, 4 and 6 hours respectively.

Ligation of the left middle cerebral artery (MCA) induced a cerebral infarct with a volume of 27 ± 1.3 mm^3 (n= 10) in inbred Balb/c mice, and of 16 ± 1.3 mm^3 (n= 12) in inbred C57BL/6 mice.

Injection of 0.2 mg Pli in Balb/c mice reduced the infarct size to 22 ± 1.0 mm^3 (n= 9) (p= 0.006 vs saline). Similar decreases were observed when the Pli injection was given 15 min before or 15 min after ligation of the MCA (Table 1). In C57Bl/6 mice, injection of 0.2 mg Pli reduced the infarct size to 10 ± 1.2 mm^3 (n= 12) (p= 0.004 vs saline).

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CLAIMS

1. Use of compounds that reduce α_2-antiplasmin in vivo, for the preparation of a therapeutical composition for the treatment of focal cerebral ischemic infarction (ischemic stroke).

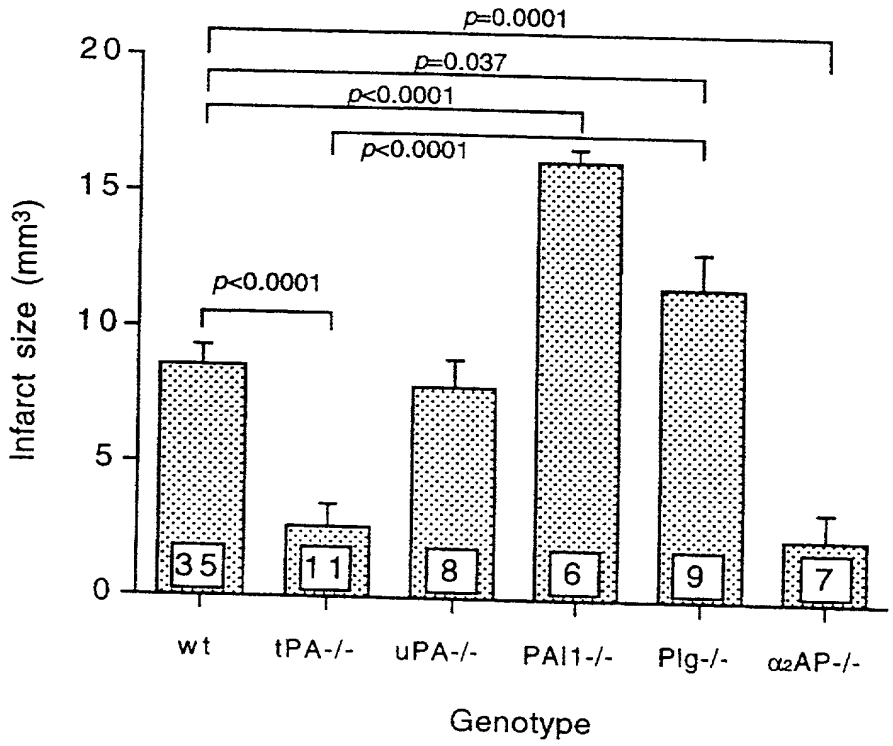
2. The use according to claim 1, wherein the compounds reduce the circulating α_2-antiplasmin concentration.

3. The use according to claim 1, the compounds reduce the circulating α_2-antiplasmin activity.

4. The use according to claims 1-3, wherein the compounds are α_2-antiplasmin neutralizing antibodies or derivatives thereof.

5. The use according to claim 4, wherein the derivatives are Fab fragments or ScFv fragments.

6. The use according to claims 1-3, wherein the compounds are α_2-antiplasmin neutralizing compounds selected from plasmin, mini-plasmin (lacking the first four kringles) or micro-plasmin (lacking all five kringles).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Genotype

FIG. 1

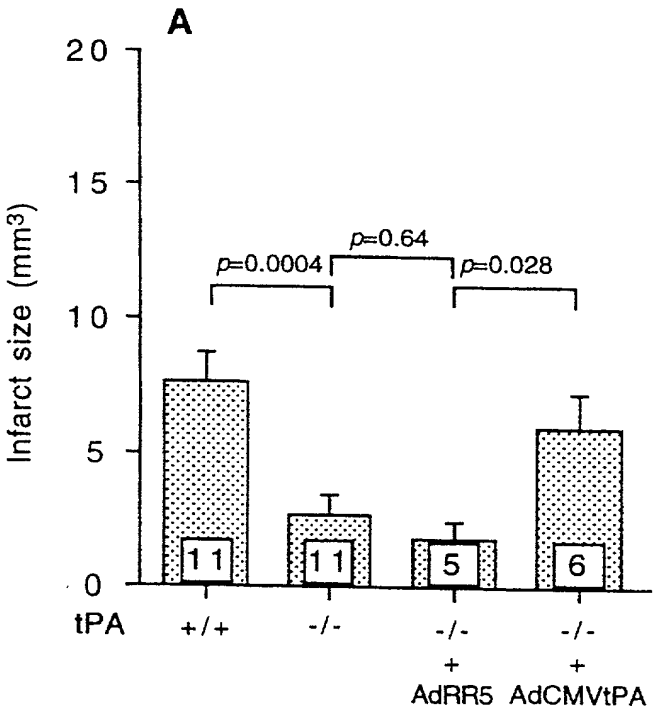
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Fig. 2A

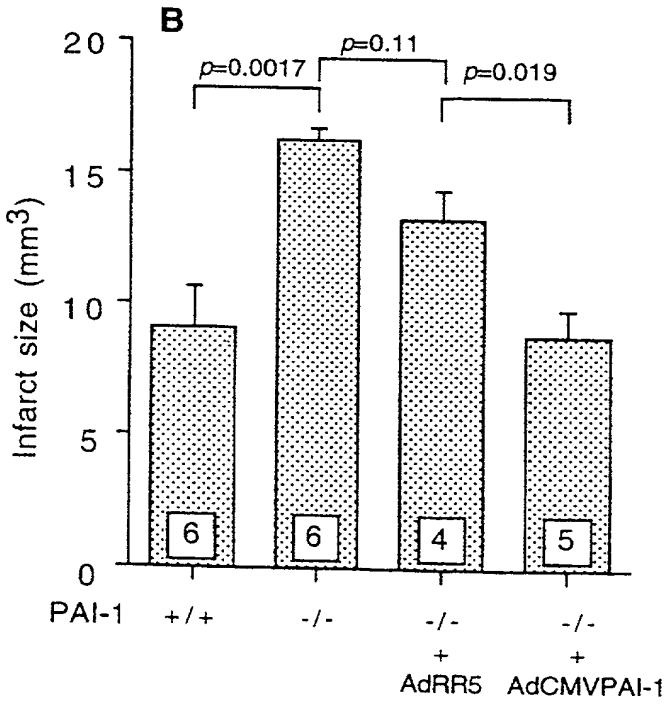
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Fig. 2B

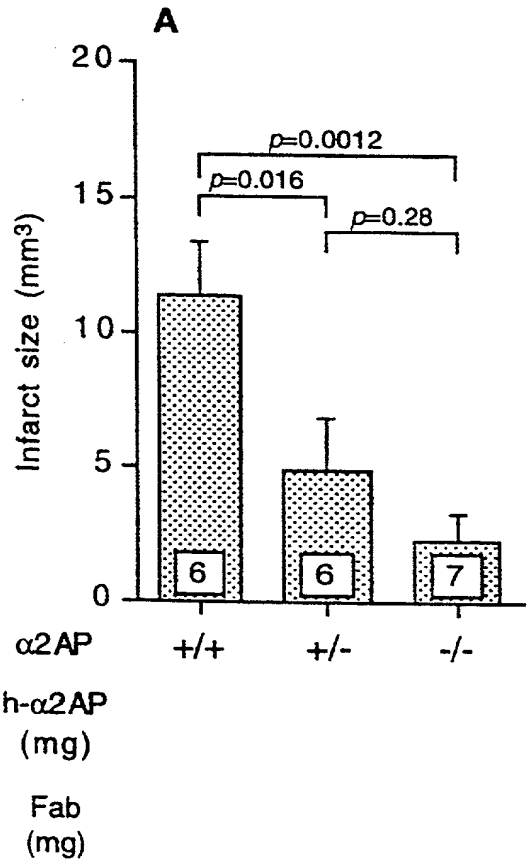
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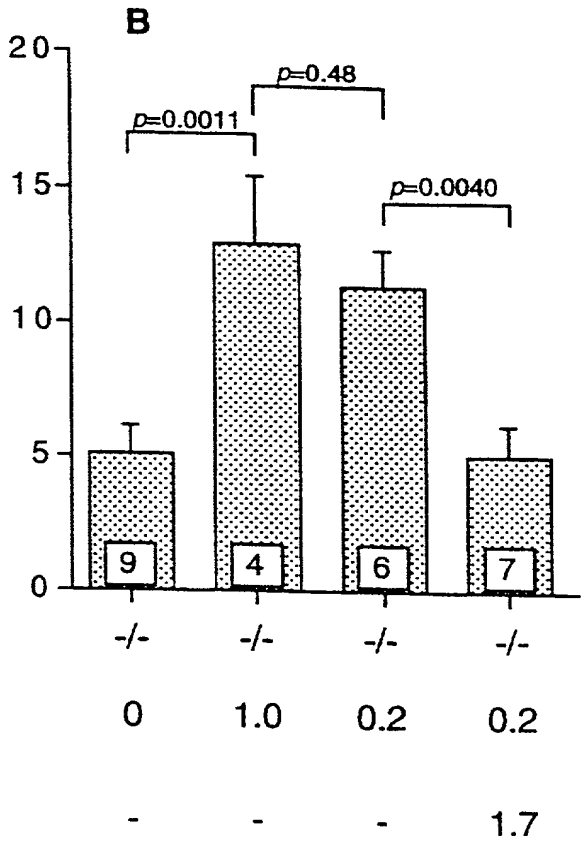
Fig. 3A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-
-
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Fig. 3B

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Use of compounds that reduce alpha2-antiplasmin in vivo for the preparation of composition for the treatment of ischemic stroke, the specification of which

(check one)

☐ is attached hereto.

☒ was filed on September 24, 1999 as PCT/EP99/07405 as

Application Serial No. 09/806,178 received March 28, 2001

and was amended on March 28, 2001 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a)

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
98203280.7 (Number)	Europe (Country)	29 September 1998 (Day/Month Year Filed)	☒ Yes	☐ No
99202004.0 (Number)	Europe (Country)	22 June 1999 (Day/Month Year Filed)	☒ Yes	☐ No
(Number)	(Country)	(Day/Month Year Filed)	☐ Yes	☐ No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (*list name and registration number*)

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